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14. ABSTRACT Operation Brain Trauma Therapy (OBTT) is a multi-center pre-clinical drug screening consortium testing therapies in multiple animal models of traumatic brain injury (TBI) with the goal of identifying the best therapies for clinical trials. OBTT is also evaluating serum biomarkers of TBI across its models. Screening of therapies is carried out at three sites using three distinct rat models, i.e., parasagittal fluid percussion injury (FPI, University of Miami), controlled cortical impact (CCI, University of Pittsburgh), and penetrating ballistic-like brain injury (PBBI, WRAIR). The serum biomarkers GFAP and UCH-L1 are being serially assessed in all models (Banyan). Biomarker development is also ongoing in a large animal (micropig) FPI model (Virginia Commonwealth University) with parallel studies. The most promising drug will be tested in the micropig model to advance a therapy up the phylogenetic scale. OBTT's work is ongoing as it completes year 3 of operation. OBTT is currently screening its 6 <sup>th</sup> drug, has studied >600 rats, and assessed ~1000 biomarker samples.					
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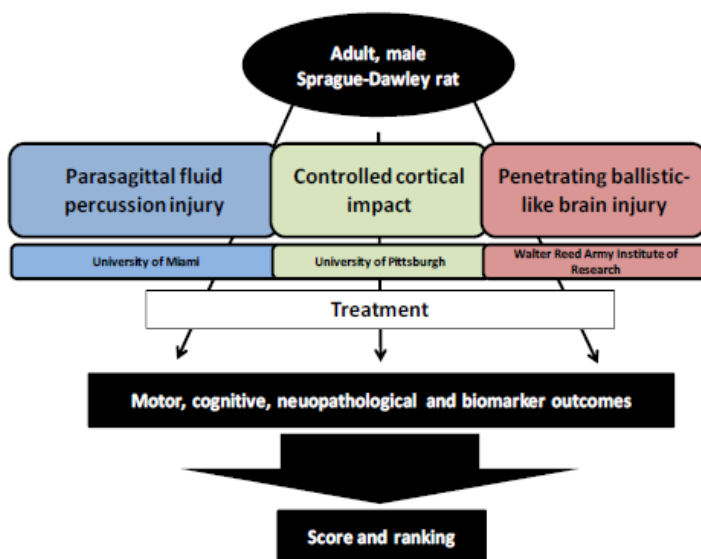
## INTRODUCTION

As outlined in the original grant proposal and in the prior reports, Operation Brain Trauma Therapy (OBTT) is a unique multi-center, pre-clinical, drug screening and brain injury biomarker development consortium for the ultimate translation of the best potential drugs to clinical trials in traumatic brain injury (TBI, **Figure 1**). OBTT includes investigators at the Safar Center for Resuscitation Research (University of Pittsburgh School of Medicine, Patrick Kochanek, MD, PI; C. Edward Dixon, Co-I), the Miami Project to Cure Paralysis, (University of Miami School of Medicine, W. Dalton Dietrich, site PI; Helen Bramlett, Co-I), the Neuroprotection program at WRAIR (Frank Tortella, site PI; Deborah Shear, PhD, and Kara Schmid, PhD, Co-Is), Virginia Commonwealth University (John Povlishock, PhD, site PI) and Banyan Biomarkers (Ronald Hayes, PhD site PI) and Kevin Wang, PhD

(University of Florida). Three rodent models (controlled cortical impact [CCI], parasagittal fluid percussion injury [FPI], and penetrating ballistic-like brain injury [PBBI]) are used in Pittsburgh, Miami, and WRAIR, respectively, for primary drug screening with the most promising candidate tested in a micropig model at Virginia Commonwealth University. Additional secondary screening of the most promising drugs is also carried out in more complex rodent models with polytrauma, hemorrhage or advanced monitoring, as deemed appropriate. **The principle concept and overall hypothesis of OBTT is that clinical TBI is a heterogeneous disease process that involves multiple brain injury phenotypes and that success of an agent tested across multiple established TBI models using an approach with unprecedented rigor and blinding across centers will identify the best candidates for success in clinical trials.** Two types of drugs are screened, 1) low hanging fruit (drugs already FDA approved for other uses, or otherwise ready for clinical translation) and 2) higher risk but potentially high reward more novel therapies. However, drugs in the latter category should have at least some track record of success in experimental TBI.

## BODY

### Models used for primary screening therapies in OBTT



**Figure 2.** Overview of the three sites involved in Primary Screening of therapies in established rat models of TBI.

Administrative overview of accomplishments in year 3 of funding  
 Safar Center for Resuscitation Research  
 (Patrick M. Kochanek, MD, overall PI)

Year 3 was another highly productive one for the OBTT consortium. The overall approach to primary screening of therapies is shown in **Figure 2**. **We have studied five therapies (Nicotinamide, Erythropoietin [EPO], Cyclosporine-A [CsA], Simvastatin, and Levetiracetam [Keppra]) across all three centers and models in >600 rats and we are currently launching testing on the sixth therapy (Glibenclamide [Glyburide]).** We have also collected and assessed nearly 1000 serum biomarker samples across the **models and treatments**. We are also carrying out preliminary pharmacokinetic (PK) studies for our 7<sup>th</sup> therapy (Minocycline)

to optimize dosing for the consortium. We have also selected therapies 8 and 9 (NIM 811, and Edaravone), which will also be evaluated across models in year 4. For each therapy, a comprehensive review of published

studies is assembled (see operations manual). Therapy selection takes place at an annual site PIs meeting at the Congress of the National Neurotrauma Society. The dosing plan for each therapy is developed based on the literature review. **For each agent, 4 experimental groups have been used in primary screening, namely, sham, injury plus vehicle, and injury plus treatment at two different doses.** The Morris water maze (MWM) is used to assess cognitive outcome and is the primary outcome parameter across sites. Motor testing is also carried out at each site, but varies depending on the model. In addition, lesion volume and hemispheric and/or cortical tissue loss are also assessed at each site. However, the drug, dose, treatment regimen, and biomarker sampling is identical between sites. **Table 1** shows the outcome scoring matrix that is used across sites for scoring in Primary Screening in OBTT. **Our work has garnered considerable attention and very positive review across the field of TBI. Notable is the fact that our work was again selected for oral presentation at the 2013 Congress of the National Neurotrauma Society and the 2013 MHSRS Conference. Among those presentations is the fact that our biomarker work was selected for oral presentation at both of those meetings. Thus far, a remarkable total of 22 abstracts have been presented by OBTT investigators (see items 2-6, 8-14, and 16-25 in the Reportable Outcomes) along with two plenary presentations by Dr. Kochanek at the National Neurotrauma Society, and the MHSRS. Finally, we are working on 6 manuscripts that will comprise a special invited issue of the *Journal of Neurotrauma* that is in preparation devoted to OBTT.** This will result in continued high visibility for the work of the OBTT consortium, reflecting the respect for the high level of rigor and quality of the investigations.

Table 1. Scoring matrix for assessment of therapeutic efficacy across models in OBTT ( ) = point value for each outcome within each model					
Site	Neuro Exam	Motor	Cognitive	Neuropathology	Serum biomarker
Miami	None	Cylinder (2) Gridwalk (2)	Hidden platform latency (2) Hidden platform pathlength (2) MWM probe (2) Working memory latency (2) Working memory pathlength (2)	Lesion volume (2) Cortical volume (2)	GFAP 24 h (1) 4-24 h Δ (1) UCH-L1 24 h (1) 4-24 h Δ (1)
Miami total	N/A	4	10	4	4
Miami Drug # Dose 1 Dose 2					
Pittsburgh	None	Beam balance (2) Beam walk (2)	Hidden platform latency (5) MWM probe (5)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4-24 h Δ (1) UCH-L1 24 h (1) 4-24 h Δ (1)
Pittsburgh total	N/A	4	10	4	4
Pittsburgh Drug # Dose 1 Dose 2					
WRAIR	Neuroscore	Rotarod (3)	Hidden platform latency (5) MWM probe (3) Thigmotaxis (2)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4-24 h Δ (1) UCH-L1 24 h (1) 4-24 h Δ (1)
WRAIR total	1	3	10	4	4
WRAIR Drug # Dose 1 Dose 2					
Grand total					
Drug # Dose 1 Dose 2					

MWM = Morris water maze; WRAIR=Walter Reed Army Institute of Research; GFAP = glial fibrillary acidic protein, UCH-L1 = Ubiquitin carboxy-terminal hydrolase L1; Δ = delta; N/A = not applicable

We also hold a monthly 1 h conference call that includes a representative from each site (we have held a total of 35 such calls since the inception of OBTT) and we have also held a very productive face-to-face investigators meeting each year, as indicated above. Therapy selection for the year is one of the agenda items each year. Dr. Kochanek also sends this report to each member of the “Therapy and Oversight Committee” and receives their input. Also, our consultants on behavior (Dr. Robert Hamm) and statistics (Dr. Stephen Wisniewski) are also appraised of our plan and they contributed recommendations. In 2012, Dr. Kochanek was invited to give plenary lectures on OBTT at the annual congress of the National Neurotrauma Society and the MHSRS. He also presented at the DoD TBI pharmacological review, on Oct 2<sup>nd</sup>, 2012, at Fort Detrick and contributed to the Pharmacology working group on Oct 3<sup>rd</sup> led by COL Salzer and Dr. Ramon Diaz-Arrastia. That document was recently published in the *Journal of Neurotrauma* and is an important summary of TBI

pharmacology for the field (Diaz-Arrastia et al, *J Neurotrauma*, Epub Ahead of Print, 2013).

## Primary screening in rodent models of TBI

An overview of the models and sites involved in Primary Screening in OBTT was provided in **Figure 2** above. Thus far, we have completed primary screening of nicotinamide, EPO, CsA, and simvastatin, are almost completed with studies of levetiracetam, and are launching glibenclamide. We are also carrying out PK studies to assess the optimal treatment regimen for Minocycline which will be drug 7 that will begin testing in Jan 2014. A complete presentation of all data from all sites for all drugs tested would be beyond the scope of this report. However, an overview of findings, to date, in primary screening are provided in the remainder of this report. **Finally, based on our progress to date, we were invited to expand our work on OBTT from 3 to 5 drugs per year. In response to this request, we submitted a proposal titled “OBTT Extended Studies” and are awaiting disposition on that additional support for our work.**

### Drug 1: Nicotinamide:

An overview of the general approach taken for drug screening in Pittsburgh is provided in **Figure 3**. This approach was used for nicotinamide at all three sites with minor modifications based on the specific outcomes at each site shown in Table 1.

Table 2 shows the finalized scoring matrix for assessment of nicotinamide.

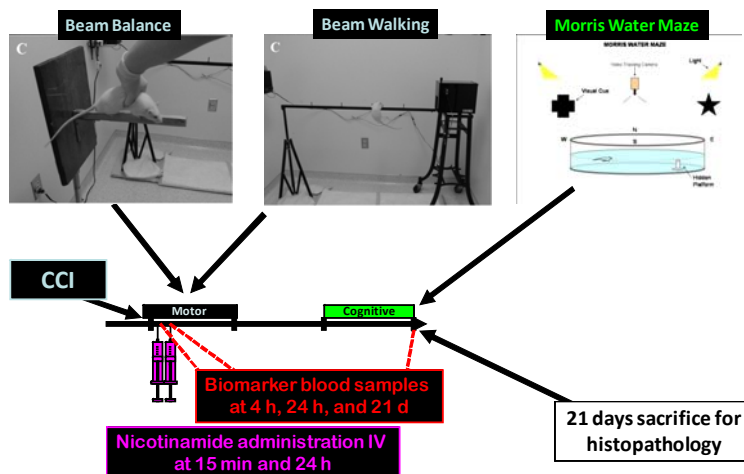


Figure 3. Protocol overview for primary screening in rats in Pittsburgh.

Table 2. Scoring matrix for assessment of therapeutic efficacy across models in OBTT ( ) = point value for each outcome within each model <b>NICOTINAMIDE—FINAL</b>						Model & Overall Total
Site	Neuro Exam	Motor	Cognitive	Neuropathology	Serum biomarker	
Miami	None	Cylinder (2) Gridwalk (2)	Hidden platform latency (2) Hidden platform pathlength (2) MWM probe (2) Working memory latency (2) Working memory pathlength (2)	Lesion volume (2) Cortical volume (2)	GFAP 24 h (1) 4-24 h Δ (1) UCH-L1 24 h (1) 4-24 h Δ (1)	
Miami total	N/A	4	10	4	4	
Miami Drug #						
Dose 1		0,0	0,0,0,0,0	0,0	-0,5,0,0,0	-0,5
Dose 2		0,0	0,0,0,+1,0	0,0	0,-0,5,0,0	+0,5
Pittsburgh	None	Beam balance (2) Beam walk (2)	Hidden platform latency (5) MWM probe (5)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4-24 h Δ (1) UCH-L1 24 h (1) 4-24 h Δ (1)	
Pittsburgh total	N/A	4	10	4	4	
Pittsburgh Drug #						
Dose 1		0,0	-2,5,0	0,0	0,0,0,0	-2,5
Dose 2		+1,0	0,0	0,+2	0,+0,5,0,0	+3,5
WRAIR	Neuroscore	Rotarod (3)	Hidden platform latency (5) MWM probe (3) Thigmotaxis (2)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4-24 h Δ (1) UCH-L1 24 h (1) 4-24 h Δ (1)	
WRAIR total	1	3	10	4	4	
WRAIR Drug #						
Dose 1	0	0	0,0,0	0,0	0,0,-0,5,0	-0,5
Dose 2	0	0	0,0,0	0,0	+0,5,+0,5,-0,5,0	+0,5
Grand total						
Drug #	0	0	-2,5	0	-1	-3,5
Dose 1	0	+1	+1	+2	+0,5	+4,5

Overall Nicotinamide

Low dose -3.5

High dose +4.5

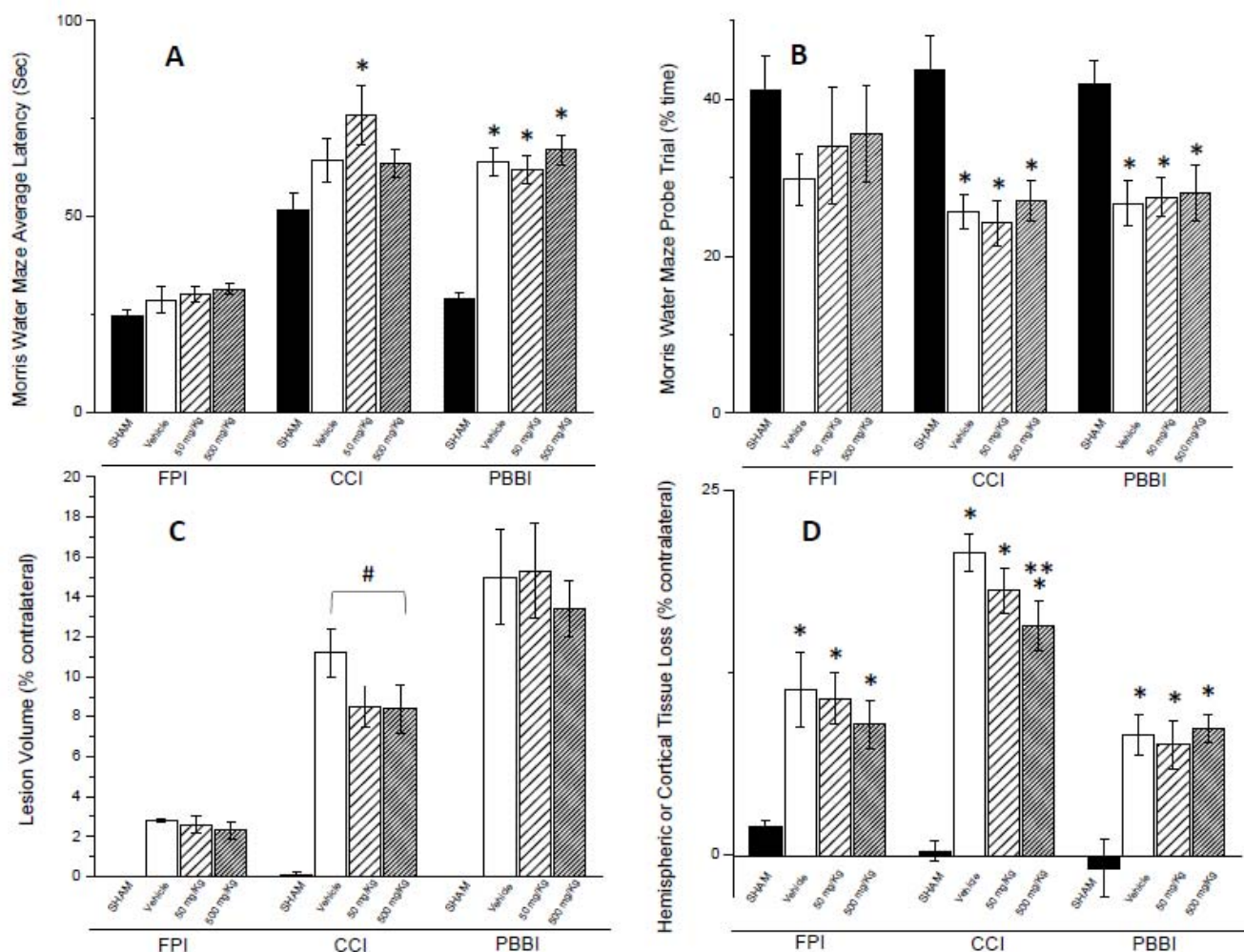
Largest positive model effect for high dose +3.5 in CCI

**Table 2.** Outcome scoring matrix results for Drug 1, nicotinamide after primary screening in rats in OBTT. Overall, nicotinamide, had a modest benefit only at high dose. It was seen largely in the CCI model and the greatest contributor to that effect was tissue sparing. Benefits on cognitive outcome were sparse—with negative effects at low dose and a benefit at high dose on only 1 outcome (working memory) in only a single model (FPI).

report, **Figure 4, A-D** provides a synopsis of some of the key findings for nicotinamide across the models.

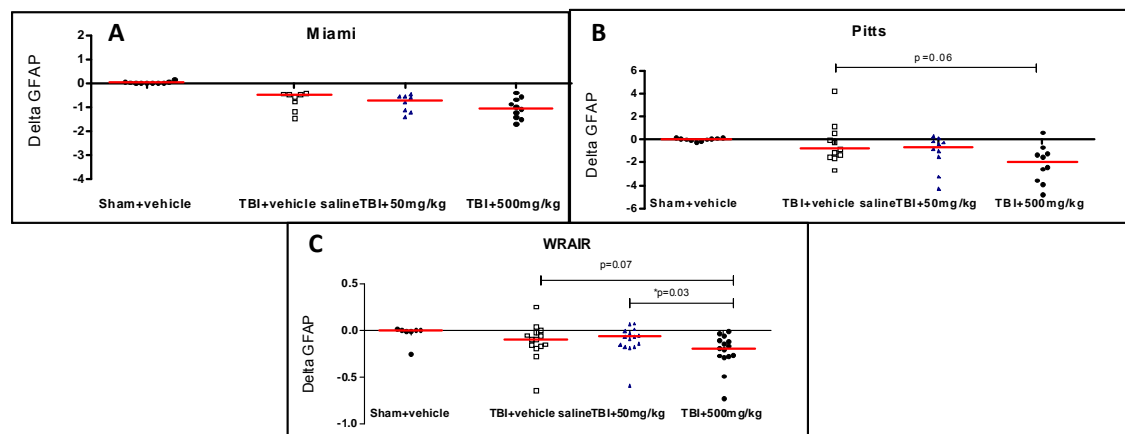


Probably the most impressive effect of nicotinamide was its effect on tissue sparing which was significant in the CCI and showed trends in FPI. Parallel to those findings, we saw a significant reduction of serum GFAP in PBBI and with strong trends in the CCI model (**Figure 5A-C**), suggesting that biomarkers may have potential to



**Figure 4 A-D.** Montage of 4 outcomes in screening of nicotinamide in OBTT in the FPI model (Miami) the CCI model (Pittsburgh) and PBBI model (WRAIR). These graphs provide an overview of drug effects across models and show the level of severity produced by each model. (A) Deficit in MWM performance in the hidden platform paradigm was modest in FPI and more substantial in CCI and PBBI. Surprisingly, low dose nicotinamide worsened MWM latency in CCI. (B) Probe trial showed again a modest deficit on the FPI model with trends toward benefit from nicotinamide. In CCI and PBBI robust deficits were seen, but neither dose improved performance. (C) Damage was modest in FPI vs. CCI or PBBI as assessed by lesion volume. There was a trend toward reduced lesion volume for both doses in CCI ( $P = 0.07$ ). (D) Tissue loss normalized for contralateral cortical volume in FPI and for hemispheric volume in CCI and PBBI showed a **significant reduction in CCI** (\*\* $P < 0.05$  for high dose vs. vehicle). A trend toward a reduction in tissue loss was also seen in the FPI model. **This approach is being used for all drugs in OBTT.**

assess tissue loss. Disappointing however, with nicotinamide was the fact that there were no major beneficial



**Figure 5 A-C.** Delta GFAP levels (24 h post TBI minus baseline value) in (A) FPI, (B) CCI, and (C) PBBI models for treatment with nicotinamide vs sham or TBI+vehicle treatment in OBTT. Treatment with nicotinamide produced trends toward benefit in serum GFAP levels across models. In CCI this reached a  $P$  value of 0.06. In PBBI, this effect was significant for high dose vs vehicle ( $P < 0.05$ )

effects on cognitive function across models. As is obvious from our scoring matrix (**Table 1**), cognitive function is logically the most important outcome and generates the most potential points in each model in OBTT. Looking back at the literature on

nicotinamide, tissue sparing is the most consistently reported finding along with benefit on motor function; cognitive outcome benefits are rarely reported. Thus, our findings in OBTT suggest that the literature may be somewhat overstated, although our findings were in the same direction. Our data also suggest that biomarkers, notably GFAP are worthy of continued use in our OBTT design. Our findings with nicotinamide also suggest that with tissue sparing in some models, it might be useful in combination therapy. That possibility is one we are considering as an option in future years if no single therapy appears highly effective. For example, nicotinamide plus a cognitive enhancing agent might be logical.

## Drug #2, Erythropoietin (EPO):

A search of *PubMed* before launching our screening studies revealed 28 publications showing benefit of EPO or its analogs in rodent TBI models and identified an ongoing single center clinical trial.<sup>1-28</sup> A pleiotropic cytokine involved in erythropoiesis, EPO has many effects that could be important in TBI such as anti-excitotoxic, anti-apoptotic, antioxidant, and anti-inflammatory actions, stimulation of neurogenesis

/angiogenesis, and protection of mitochondria. The mechanisms of benefit are unclear. Although classical EPO receptors are seen in many cell types in the CNS, they are up-regulated by hypoxia, and EPO receptor null mice have a worse outcome than wt after CCI, surprisingly, EPO receptors do not appear to be needed to mediate the benefit of exogenously administered EPO. In the 24 studies, species included rats and mice and models included CCI, FPI, impact acceleration, closed head injury, Feeney weight drop, and TBI plus hemorrhagic hypotension. Studies in large animal models, however, were not identified. **Based on the literature, we chose doses of 5000 U/kg and 10,000 U/kg as a single IV bolus at 15 min after TBI.** 5000 U/kg had the

Table 3. Scoring matrix for assessment of therapeutic efficacy across models in OBTT ( ) = point value for each outcome within each model EPO						Model & Overall Total
Site	Neuro Exam	Motor	Cognitive	Neuropathology	Serum biomarker	
Miami	None	Cylinder (2) Gridwalk (2)	Hidden platform latency (2) Hidden platform pathlength (2) MWM probe (2) Working memory latency (2) Working memory pathlength (2)	Lesion volume (2) Cortical volume (2)	GFAP 24 h (1) 4-24 h Δ (1) UCH-L1 24 h (1) 4-24 h Δ (1)	
Miami total	N/A	4	10	4	4	
Miami Drug #						
Dose 1		0,0	0,0,0,-2,0	0,0	0,0,0,0	-2,0
Dose 2		0,0	0,0,0,0,0	0,0	0,0,0,0	0
Pittsburgh	None	Beam balance (2) Beam walk (2)	Hidden platform latency (5) MWM probe (5)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4-24 h Δ (1) UCH-L1 24 h (1) 4-24 h Δ (1)	
Pittsburgh total	N/A	4	10	4	4	
Pittsburgh Drug #						
Dose 1		0,0	-2,5,0	0,0	0,0,0,0	-2,5
Dose 2		0,0	-2,5,0	0,0	0,0,0,0	-2,5
WRAIR	Neuroscore	Rotarod (3)	Hidden platform latency (5) MWM probe (3) Thigmotaxis (2)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4-24 h Δ (1) UCH-L1 24 h (1) 4-24 h Δ (1)	
WRAIR total	1	3	10	4	4	
WRAIR Drug #						
Dose 1	0	0	0,+1,5,0	0,0	0,0,0,0	+1,5
Dose 2	0	0	0,+1,5,0	0,0	0,0,0,0	+1,5
Grand total						
Drug #	0	0	-3,0	0	0	-3,0
Dose 1	0	0	-1,0	0	0	-1,0
Dose 2						

Overall EPO

Low dose -3.0

High dose -1.0

Largest positive model effect +1.5 in PBBI for either dose

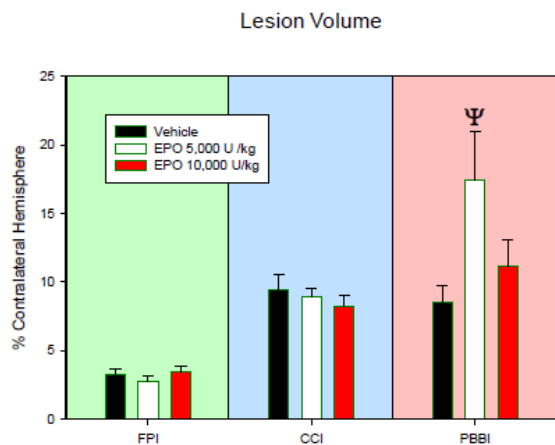
**Table 3.** Outcome scoring matrix results for Drug 2, EPO after primary screening in rats in OBTT. Overall, EPO, had modest detrimental effects in the FPI and CCI models and equivocal effects in PBBI. Although there was a mild benefit on probe trial in PBBI, neuropathology in that model showed a nearly significant expansion of lesion volume ( $P = 0.057$ , see **Figure 6**). The lack of benefit of EPO was surprising given its publication record, however, our findings were consistent and all experiments were rigorously done in a blinded fashion.

most literature support—the higher dose tested dose response. Therapeutic window is controversial; some studies suggest benefit with first dose as late as 24 h. The most complete study of time window identified 6 h as the latest effective time point. **Again, at each site, we used four groups, sham, TBI + vehicle, and TBI + treatment at low and high doses** with an overall sample size of ~10 rats per group in each model.

**The overall scoring sheet for EPO is provided in Table 3.** Surprisingly, unlike nicotinamide, where we noted some benefit on at least one or two outcomes in each model, we did not detect robust beneficial effects of EPO across outcomes in the consortium.

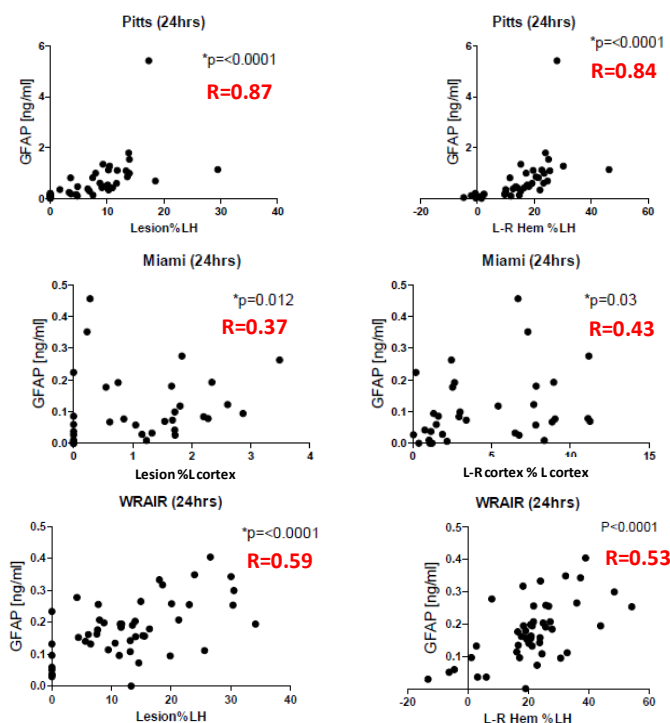


Indeed, in general effects were modest. However, mostly deleterious effects were seen. **Surprisingly, in both the FPI and CCI models, we observed deleterious effects on one aspect of cognitive function.** Table 3



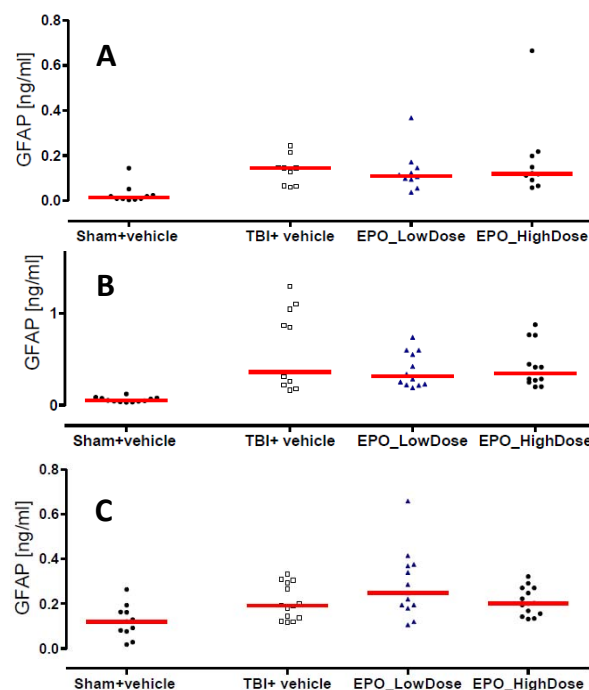
**Figure 6.** Lesion volume (as % contralateral hemisphere in CCI and PBBI and as % contralateral cortex in FPI) at 21d after TBI across the OBTT consortium. Surprisingly, no reduction of lesion volume was seen at either dose in any model. Indeed, there was a trend toward increased lesion volume for the 5000 U/kg dose in the PBBI model with a significant ANOVA and  $\Psi P=0.057$  for treated vs. vehicle on post hoc testing.

only identifies what was statistically significant for EPO across the models. Some other effects also suggested by our results included a trend toward expansion of contusion volume in the PBBI model that approached significance ( $P = 0.057$  vs. vehicle) (**Figure 6**). This was surprising given the fact that effects on tissue sparing have been reported by other groups. With regard to serum biomarkers in our studies with EPO, in general, as shown in Table 3, there were no significant effects of EPO on serum biomarker levels. GFAP at 24 h has generally appeared to perform best across models in OBTT with regard to correlations with lesion volume in our consortium. This is illustrated in **Figure 7**. Building on that work, and consistent with our findings on other outcomes, we did not see any beneficial effect of EPO treatment on 24 h GFAP levels across models (**Figure 8**). We saw some complex effects of EPO on serum UCH-L1 levels in the CCI model that were discussed in last year's report.



**Figure 7.** Correlations between 24 h serum GFAP levels and lesion volume (left sided panels) or hemispheric tissue loss (right sided panels) across the three models CCI (Pittsburgh), FPI (Miami), and PBBI (WRAIR). GFAP showed the best correlation with tissue loss in OBTT.

Our overall conclusion with EPO is that it was surprisingly ineffective across our OBTT consortium either overall or considering its effects in any of the three individual models. A limitation to our testing with EPO is that we tested only single dosing



**Figure 8.** Effect of EPO on serum GFAP levels at 24 h after TBI. Consistent with our other outcomes, despite increases in GFAP after injury across models, no effect of treatment was seen on this biomarker in (A) FPI, (B) CCI, or (C) PBBI in OBTT.

approach, namely, at 15 min after TBI. Some have suggested dosing for several days. However, supporting our approach, early post-injury dosing has shown success in many reports. Nevertheless, multiple/chronic dosing might show greater effects and might merit examination in pilot studies. Our overall findings with EPO however, dampened enthusiasm for additional studies with this agent, given the many potential drugs that merit testing using the OBTT mechanism. **Abstracts #17, 19, 21, 22, 24, and 25 in Reportable Outcomes address our work on EPO.**

**Drug #3. Cyclosporine-A (CsA):** CsA is a low hanging fruit drug in widespread clinical use as an immunosuppressant. Inhibition of mitochondrial permeability transition pore opening is suggested to confer benefit in TBI by preserving mitochondrial function and reducing ROS. Calcineurin inhibition may also benefit learning/memory by blocking its phosphatase activity. Immunosuppressive effects, also mediated by calcineurin inhibition, may also confer benefit (or side effects). 17 studies in pre-clinical TBI models were identified on *PubMed* (16 positive).<sup>29-45</sup> Multiple histological outcomes were benefited (axonal injury, lesion volume) in multiple labs. Surprisingly, there are few studies of CsA on behavior after TBI—two studies show benefit on motor outcomes, and one on MWM. Most studies were carried out in impact acceleration or CCI, with a few in FPI. All but 3 were carried out in rats, with one in mice, piglets, and ewe. Most work was done in males. There are many studies of dose response, route of administration, therapeutic window, and brain tissue levels. IV dosing is preferred in OBTT and available for CsA. Early work showed limited BBB passage. While

that is true in uninjured brain, data in impact acceleration in rats show that brain tissue levels of CsA after 20 mg/kg mirror those seen after a 10mg/kg intrathecal dose. Most studies show efficacy with 10-20 mg/kg. Curiously, the only study showing benefit on cognitive outcome used low doses of 0.675mg/kg or 18.75mg/kg. In other studies, 1 or 3mg/kg were of little efficacy on histology. High doses of 150mg/kg were also not effective. Therapeutic window studies suggest that 15min is better than 1h with some efficacy to 8h. Some studies used a second dose at 24h. Maximal effect on permeability transition was seen at 0.5-1.0  $\mu$ M but extrapolation of in vitro studies is complex.<sup>46-48</sup> A number of studies have been done on rodent CsA PK.<sup>49-53</sup> The terminal  $T_{1/2}$

Table 4. Scoring matrix for assessment of therapeutic efficacy across models in OBTT ( ) = point value for each outcome within each model <b>CsA</b>						Model & Overall Total
Site	Neuro Exam	Motor	Cognitive	Neuropathology	Serum biomarker	
Miami	None	Cylinder (2) Gridwalk (2)	Hidden platform latency (2) Hidden platform pathlength (2) MWM probe (2) Working memory latency (2) Working memory pathlength (2)	Lesion volume (2) Cortical volume (2)	GFAP 24 h (1) 4-24 h $\Delta$ (1) UCH-L1 24 h (1) 4-24 h $\Delta$ (1)	
Miami total	N/A	4	10	4	4	
Miami Drug #						
Dose 1		0,0	0,1,0,0,0	0,2	0,0,0,0	3
Dose 2		0,0	0,1,0,0,-1	0,0	0,0,0,0	0
Pittsburgh	None	Beam balance (2) Beam walk (2)	Hidden platform latency (5) MWM probe (5)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4-24 h $\Delta$ (1) UCH-L1 24 h (1) 4-24 h $\Delta$ (1)	
Pittsburgh total	N/A	4	10	4	4	
Pittsburgh Drug #						
Dose 1		-1,0	0,0	0,0	0,0,0,0	-1
Dose 2		-1,0	-2,5,0	0,0	0,0,0,0	-3.5
WRAIR	Neuroscore	Rotarod (3)	Hidden platform latency (5) MWM probe (3) Thigmotaxis (2)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4-24 h $\Delta$ (1) UCH-L1 24 h (1) 4-24 h $\Delta$ (1)	
WRAIR total	1	3	10	4	4	
WRAIR Drug #						
Dose 1	0	0	0,0,0	0,0	0,0,0,0	0
Dose 2	0	0	0,0,0	0,0	0,0,0,0	0
Grand total						
Drug #	0	-1	1	2	0	2.0
Dose 1	0	-1	-2.5	0	0	-3.5
Dose 2						

**Overall CsA**

**Low dose +2.0**

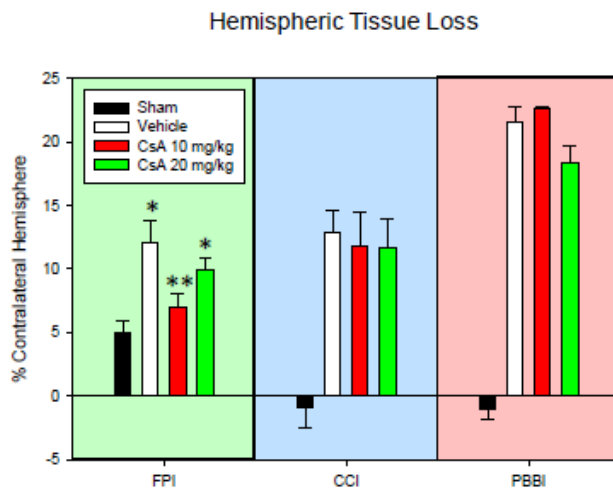
**High dose -3.5**

**Largest positive model effect +3.0 in FPI for low dose**

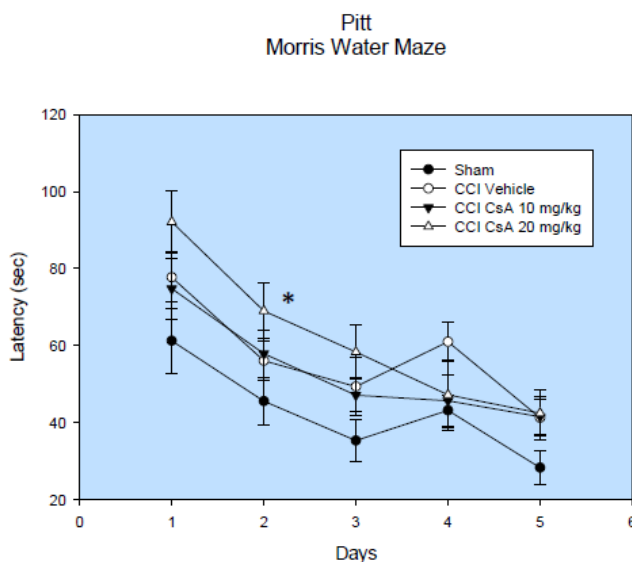
**Table 4.** Outcome scoring matrix results for Drug 3, CsA after primary screening in rats in OBTT. **CsA was unique thus far among drugs in OBTT in that effects were highly model dependent.** In FPI, our mildest insult, CsA showed modest benefit at low dose, and no toxicity. In CCI, CsA showed modest deleterious effects at both doses and mild toxicity. In the more severe PBBI, both CsA at high dose, and its vehicle (cremophor) were toxic and associated with an increase in mortality rate and no benefit on outcome in survivors. Please see text for details.

of CsA in rats of 7.5-12h suggests that q24 h dosing is reasonable at 10 or 20 mg/kg. Plasma levels will likely be  $>1\mu$ M with 10mg/kg at 24h. Brain penetration/kinetics in TBI is complex. Data exist on total (not free) brain levels in naïve rats ( $\sim 2\mu$ M in brain 45min after a 20 mg/kg IV). Lemaire showed 0.85 and 9.9  $\mu$ M 2h after 10 and 30 mg/kg IV. Tanaka showed 6 and 30 mg/kg CsA IV had 24h troughs of  $\sim 0.3$  and  $\sim 2\mu$ g/mL (0.5  $\mu$ M=0.6  $\mu$ g/mL). In controls, CsA shows saturable brain distribution with dose-level nonlinearity at  $>3$ mg/kg IV in rats; levels go up in brain disproportionate to dose. CNS toxicity has been seen in rats at 50 mg/kg likely for this reason. Overall, 10 and 20 mg/kg IP are most supported. It is unclear what percent of the 24h dosing interval will produce free levels  $>0.5$ -1  $\mu$ M in injured brain. Without injury, total but not free levels will likely exceed this level for most of the 24h interval. IV dosing will likely yield higher levels than prior TBI studies using IP dosing given low IP bioavailability and TBI will increase brain penetration. **Thus, we tested 10 or 20 mg/kg IV infused over 5 min at 15 min and 24 h after injury across models in OBTT. The overall scoring sheet for CsA is provided in Table 4. Unlike other drugs tested thus far, CsA as used in OBTT showed great model dependence.**

Although data analysis is ongoing, CsA showed some benefit in the mildest insult FPI, but modest deleterious effects on motor and cognitive function in CCI and mild toxicity (2 rats died; 2 had seizures). In the most severe model, PBBI, both CsA and its vehicle (cremophor) showed considerable toxicity at high dose (29% mortality and similar mortality in the vehicle group) and no benefit on any outcome. Some of these effects in individual models, highlighting the model dependence of CsA across OBTT, are shown in **Figures 9 and 10**.



**Figure 9.** Brain tissue loss at 21d after TBI across OBTT: effects of CsA. Note, for FPI this represents cortical tissue loss (as % of contralateral cortex) while in CCI and FPI it is hemispheric tissue loss. In all three models, there is significant tissue loss. In FPI, low dose CsA produced a substantial beneficial effect of ~50% reduction in cortical tissue loss (\* $P < 0.05$  vs vehicle), with a trend toward benefit at high dose. In contrast, no differences in hemispheric tissue loss between groups were seen in either CCI or PBBI. This finding highlights the considerable model dependence of CsA in OBTT.



**Figure 10.** In contrast to the beneficial effects of CsA in the FPI model, in the more severe CCI model, high dose CsA showed deleterious effects on MWM performance, and as indicated in the text, showed toxicity in CCI and PBBI. This suggests that CsA or its analogs might be useful to pursue in mild TBI, but caution is in order in severe TBI. \* $P < 0.05$  vs sham only for the 20 mg/kg CCI group.

Presentation of all of the outcome data is beyond the scope of this report.

Lack of a benefit on tissue sparing in CCI and deleterious effects on behavior were surprising. However, few pre-clinical studies have assessed behavioral outcomes with CsA in TBI. Toxicity in PBBI was unanticipated. One possibility is that we used IV dosing, contrasting the IP dosing in most other work. IP absorption of CsA is erratic and it is possible that high levels show toxicity in models where there is a high level of BBB permeability. Our work also suggests that differences between patients in the amount of BBB injury might make the response to CsA treatment variable—from benefit to toxicity, and could make this agent challenging to dose. In PBBI, the vehicle was also toxic. Assessment of brain tissue levels across models could be helpful. Our findings suggest that CsA or some of the more novel analogs of CsA with potentially less toxicity, such as NIM 811 or Neurovive might be logical to test. This might be particularly true in mild TBI—a finding which could be important to the

Army. We plan to consider studying NIM 811, and submitted a request to Novartis for this drug for OBTT; they approved our request. We will address MTA related issues and test NIM 811 in 2014. It is a non-immunosuppressant CsA analog reported to potentially reduce mitochondrial permeability transition pore opening. It might thus be promising in TBI as suggested in recent reports from the Sullivan group in Kentucky. **We believe that our studies with CsA exemplify the value of OBTT—namely, the critical importance of testing therapies in multiple models given the myriad TBI phenotypes and severities in humans. Abstracts #16, 18, 20, 22, 23, and 25 in Reportable Outcomes address our work on CsA.**

#### Drug #4. Simvastatin

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG) reductase inhibitor Simvastatin reduces serum cholesterol but also inhibits neuro-inflammation and possible effects on brain edema, Akt, CBF and trophic factor production. A total of 15 studies were identified with Simvastatin in TBI.<sup>54-68</sup> Oral dosing reduced CA3 cell death and improved MWM performance after CCI in rats. The MWM findings were limited to benefit on probe trial. Simvastatin showed greater benefit than Atorvastatin. Both are FDA approved and are, thus, low hanging fruit candidates. Sierra et al<sup>69</sup> compared 9 statins with regard to their BBB penetration, HMG CoA reductase inhibition, and protection vs. neuro-degeneration from Tau and concluded that Simvastatin was best. Thus, taking all of this information into consideration, Simvastatin was selected for testing by OBTT.

Mahmood et al<sup>55, 56</sup> in the group of Dr. Michael Chopp reported benefit of Simvastatin on motorscore after CCI in female Wistar rats. A reduction in CA3 hippocampal neuronal death was also seen. The Chopp group also reported a reduction in TUNEL staining in injured brain after CCI in rats with Simvastatin therapy.<sup>58,59</sup> Usually, a dose of 0.5 or 1 mg/kg daily beginning on day 1 and continued for 14 days was used; a dose of 1 mg/kg was generally optimal. In studies by other groups, Chen et al<sup>60</sup> used a weight drop model in rats and higher Simvastatin doses, namely, 37.5 mg/kg PO at 1 h and 6 h. Benefit on Rotarod, % brain water and cytokines was reported. Beziaud et al<sup>66</sup> also used 37.5 mg/kg at 1 h and 6 h after FPI in rats and noted benefit on % brain water, BBB, and several inflammatory markers in brain. Abrahamson et al<sup>62</sup> reported benefit of Simvastatin (3 mg/kg PO daily, first dose at 3 h post CCI) on probe trial, but no effect on MWM latency after CCI in mice modified to express human A $\beta$ . Chauhan et al<sup>63</sup> studied CCI in mice using 2 mg/kg oral dosing—with the drug incorporated in feeds. They reported benefit on probe trial and axonal injury. Shear et al<sup>68</sup> in our group at WRAIR studied IV Simvastatin in the PBBI model in rats at doses of 0.001, 0.01, 0.1, and 1.0 mg/kg. They gave a 10 min IV infusion at 30 min and 6h post-PBBI, and every 24h to 10d (all IV). There was no benefit on Rotarod; however, it dose-dependently protected vs. cognitive deficits on MWM. Chronic IV treatment was required to show behavioral benefit. However, not all studies with Simvastatin in experimental TBI are positive. Chen et al<sup>64</sup> used the parasagittal FPI model in rats and doses of 25, 37.5, 50, 75 or 100 mg/kg PO at 1h and 6h after TBI and noted a reduction in edema, but no benefit on neuroscore, beam walking or lesion volume.

Indraswari et al<sup>65</sup> reported that Simvastatin at 1 or 5 mg/kg PO did not improve Rotarod performance after CHI in mice. Simvastatin enhances LTP.<sup>70</sup> None of the studies in experimental TBI included naïve controls treated with Simvastatin; thus it is unclear if the cognitive enhancement after TBI represents an effect specific for TBI or simply non-specific enhancement of cognitive function.

Regarding dose, timing, and route of administration for Simvastatin, oral

(gavage) dosing of between 1 and 3 mg/kg daily for up to 14 d has shown the most benefit in published studies in experimental TBI. A dose of 0.5 mg/kg has been less effective. Several studies used higher doses, however, variable results were observed. Most oral dosing studies used 14d of treatment. Given the mission of OBTT to test promising drugs using established regimens in multiple laboratories, we tested **oral gavage treatment with Simvastatin; 1 or 5 mg/kg PO with first dose at 3 h and daily doses for 14 d.**

Table 5. Scoring matrix for assessment of therapeutic efficacy across models in OBTT ( ) = point value for each outcome within each model <b>Simvastatin—Preliminary</b>						Model & Overall Total
Site	Neuro Exam	Motor	Cognitive	Neuropathology	Serum biomarker	
Miami	None	Cylinder (2) Gridwalk (2)	Hidden platform latency (2) Hidden platform pathlength (2) MWM probe (2) Working memory latency (2) Working memory pathlength (2)	Lesion volume (2) Cortical volume (2)	GFAP 24 h (1) 4-24 h $\Delta$ (1) UCH-L1 24 h (1) 4-24 h $\Delta$ (1)	
Miami total	N/A	4	10	4	4	
Miami Drug #						
Dose 1		0,1	-2,-2,0,0,0	-	-	-
Dose 2		0,1	0,0,0,0,0	-	-	-
Pittsburgh	None	Beam balance (2) Beam walk (2)	Hidden platform latency (5) MWM probe (5)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4-24 h $\Delta$ (1) UCH-L1 24 h (1) 4-24 h $\Delta$ (1)	
Pittsburgh total	N/A	4	10	4	4	
Pittsburgh Drug #						
Dose 1		0,0	0,0	-	-	-
Dose 2		0,1	0,0	-	-	-
WRAIR	Neuroscore	Rotarod (3)	Hidden platform latency (5) MWM probe (3) Thigmotaxis (2)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4-24 h $\Delta$ (1) UCH-L1 24 h (1) 4-24 h $\Delta$ (1)	
WRAIR total	1	3	10	4	4	
WRAIR Drug #						
Dose 1	0	1.5	0,0,0	-	-	-
Dose 2	0	1.5	0,0,0	-	-	-
Grand total						
Drug #	0	2.5	-4	-	-	-
Dose 1	0	3.5	0	-	-	-
Dose 2						

#### Simvastatin Behavior

Low dose -1.5

High dose +3.5

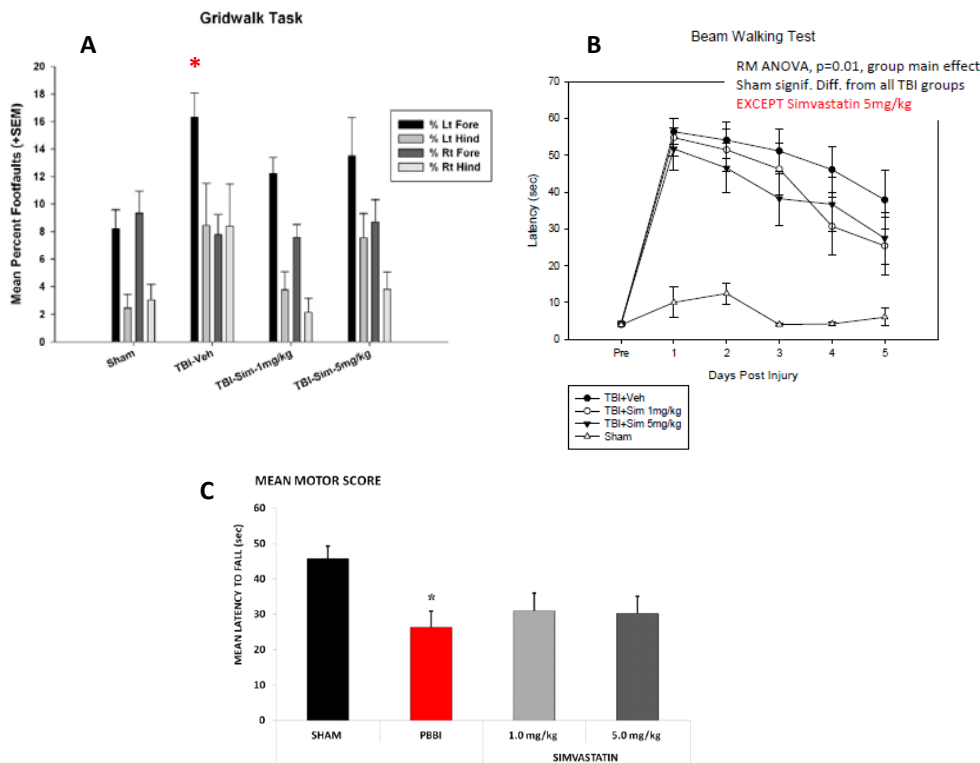


**Table 5** shows the preliminary behavioral outcomes across models for Simvastatin. We recently completed all of the injuries across the OBTT consortium and also all of the studies of behavioral outcomes after treatment with Simvastatin. This was a demanding study for the consortium with 14 days of oral gavage administration

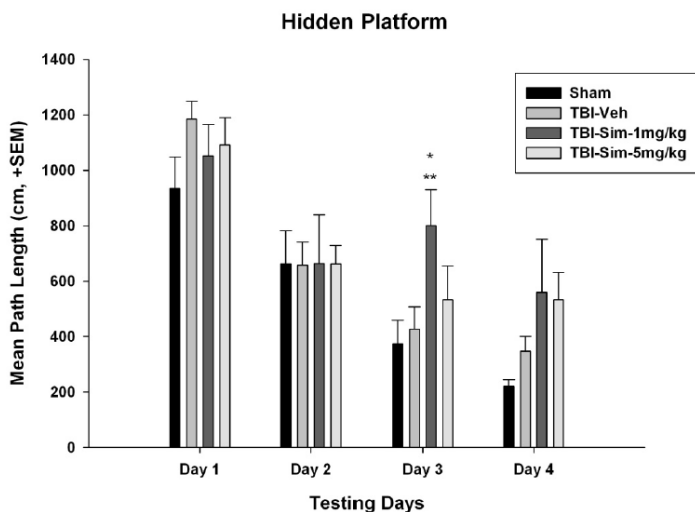
carried out in each animal at each site. Assessments of lesion volume, hemispheric tissue loss and biomarker data are ongoing. A brief discussion of the results of the behavioral studies is provided below. These results are preliminary given the fact that data analysis for this therapy is ongoing. Nevertheless, overall, as predicted by the literature, we observed some benefit on motor function, and this was seen across models. Indeed, Simvastatin represents the first therapy tested by OBTT to show some cross model benefit (in all three screening models) on any individual category of behavioral outcomes—the cross model benefit on various aspects of motor function was only mild-moderate in magnitude (**Figure 11**). However,

disappointingly, we did not detect any benefit on cognitive outcome in any of the models. And in fact, MWM

performance was actually worsened by treatment in the FPI model. This was observed despite the fact that, as indicated above, we used a demanding 14-d oral gavage treatment regimen across sites to attempt to replicate multiple studies in the published literature (**Figure 12**). Note also that all biomarker samples from these studies have been collected and shipped to Banyan and UCH-L1 and GFAP analyses are ongoing. **Of note the outcome code for behavior was only recently broken and thus our work with Simvastatin has not yet been presented at a scientific meeting. We anticipate presenting it at the 2014 NNT and MHSRS meetings.**



**Figure 11.** Preliminary results of treatment with Simvastatin for 14d at either 1 or 5 mg/kg improved motor function albeit modestly across all three rat screening models in OBTT. **(A) FPI model:** Shows the Gridwalk task after FPI. \* $P < 0.05$  vs. sham only in the vehicle group. **(B) CCI model:** In CCI, only the 5 mg/kg group was not significantly worse than sham on the beam walking task. **(C) PBBI model:** In PBBI, both the 1 and 5 mg/kg doses showed modest benefit on the Rotarod task—only the PBBI vehicle group was significantly different vs. sham. Simvastatin is the first drug to show at least some benefit (albeit modest) across all 3 screening models for any specific behavior—i.e., motor function. However, as shown in Figure 12, Simvastatin did not improve cognitive outcome and in fact exhibited some paradoxical deleterious effects in FPI.



**Figure 12.** Preliminary results of treatment with Simvastatin for 14d at 1 mg/kg increased (worsened) path length to find the hidden platform on the MWM paradigm after TBI in FPI. \* $P < 0.05$  vs. sham and \*\* $P < 0.05$  vs. TBI vehicle. This deleterious effect on cognitive outcome was not anticipated. Note the 5 mg/kg dose also showed a trend toward worse performance. Also note that the FPI model is the mildest insult of the three TBI models in OBTT, although all of the models are considered moderate-severe TBI. No beneficial or detrimental effect of Simvastatin treatment was seen in either of the other two models. Please see text for details.

hanging fruit for different reasons than most of the drugs considered by OBTT. It has a limited track record in

experimental TBI, but has three compelling features 1) it targets posttraumatic seizures by unique mechanisms compared to currently used anti-convulsants, 2) it has exceptionally low toxicity and 3) it is already in use in some clinical centers—although it is empiric and sporadic.

Levetiracetam is believed to act at least in part via potentiation of GABAergic inhibition, although some non-GABA effects are operating. It inhibits burst firing without interference with normal neuronal excitability. It has a brain-specific binding site and selectively inhibits N-type  $\text{Ca}^{++}$  channels, exhibiting brain specific effects that differ from other anticonvulsants and thus confers anti-convulsant and excitotoxic effect with little apparent extracerebral toxicity except at extremely high drug doses. There is limited pre-clinical data supporting beneficial effects of Levetiracetam in TBI. Wang et al<sup>71</sup> reported benefit in a murine CHI model with single IV doses of either 18 or 54 mg/kg at 30 min after injury and also in a second paradigm of SAH where treatment was given every 12 h for 3 d. In the CHI study, Levetiracetam was beneficial. Outcomes included Rotarod which was maximally benefited at 54 mg/kg and hippocampal cell death which was maximally benefited at 18 mg/kg (although the 54 mg/kg dose was similar). Fosphenytoin showed no benefit or detrimental effects. The rationale for testing Keppra in OBTT is thus related to several considerations 1) posttraumatic seizures, particularly with severe TBI are common (in patients) and sub-clinical status epilepticus worsens outcome in humans after severe TBI and is seen in our models, 2) the aforementioned pre-clinical study, 3) the fact that it is used by some centers as standard of care—while most use fosphenytoin, 4) the fact that there is controversy with regard to efficacy of the more commonly used acute anti-convulsant in TBI, i.e., fosphenytoin, particularly when compared to Levetiracetam, and 5) there is recent pre-clinical data in experimental TBI that suggests deleterious long-term effects of phenytoin therapy on functional and histopathological outcomes.<sup>79</sup> Thus, if OBTT were to show a clear benefit of Levetiracetam in neuroprotection and/or behavioral outcomes, it would strongly suggest the need for a clinical trial that we believe would likely be attractive and thus carried out.

**Regarding dosing, route of administration and PK, doses of 18 or 54 mg/kg IV 30 min after CHI in mice as a single dose was used in the only TBI study.** The 54 mg/kg dose was at least if not more effective versus the 18 mg/kg dose. Thus, it would be logical in OBTT, given the testing of two doses of each therapy, to test the 54 mg/kg dose and a higher dose. A 15 min dosing regimen could be readily used. Available data on PK in rats for Levetiracetam comes from several studies. Loscher et al<sup>72</sup> studied daily IP injections of 13, 27, or 54 mg/kg for 21 days. During treatment, seizure kindling was suppressed (1 h after dosing) but surprisingly, beneficial effects on seizure kindling were seen for 10 or more days after the treatment was discontinued despite the fact that the half-life of Keppra is 2-3 h in rats. Given that one of the goals of OBTT is to replicate the best available studies with a given drug, early, single dose administration with 54 mg/kg is logical. The route of administration will be IV given the availability of an IV clinical formulation.

Other studies in rats suggest that higher doses than 54 mg/kg would be worthy of exploring. Kiltgaard et al<sup>74</sup> carried out extensive studies with levetiracetam using a wide range of doses from 54 to 1700 mg/kg across many seizure paradigms such as NMDA, kainic acid, AMPA, bicuculline, picrotoxin, flumazenil, pilocarpine, and kindling, among others. Efficacy of levetiracetam varied greatly depending on the inducing agent. For example, only 7 mg/kg IP abolished pilocarpine induced seizures, 54 mg/kg vs. kainite induced seizures, 97 mg/kg vs. DMCM-induced seizures, and 170 mg/kg vs. benzodiazepine antagonist-induced seizures. Levetiracetam was not effective against bicuculline- or picrotoxin-induced seizures. Thus, there is rationale for higher doses than 54 mg/kg, and 170 mg/kg seemed logical, based on the literature. Toxicity was not seen in rats until doses of 1700 mg/kg twice daily were used; performance on Rotarod was impaired.

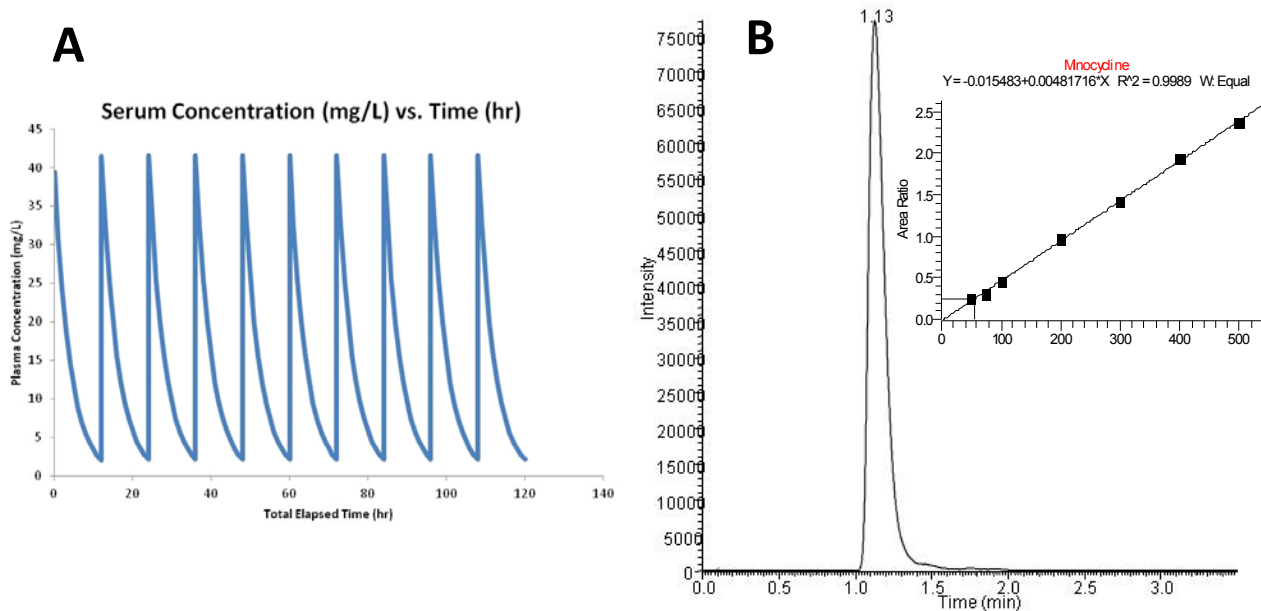
It is recommended that levetiracetam is diluted in saline before administration. In discussions with our pharmacy team, the minimum dilution should probably be 1:1. Thus, based on all of this information and on the feasibility of administration in rats (i.e., limiting the volume of fluid administration to 2 mL for each dose), **we used single 15 min post TBI dosing of either 54 or 170 mg/kg. This also limited the volume of fluid administered to 2 mL of saline (<10 mL/kg and thus clinically relevant). It was administered as a single dose at 15 min after TBI.**

**The studies with Levetiracetam in year 3 are ongoing across the OBTT consortium and all of the behavioral outcome assessments are nearly complete—over 120 studies have already been completed with this therapy. Please note, however, that the behavioral outcome code for Levetiracetam has not yet been broken because not all of the animals have completed all of the planned behavioral testing out to the final 21 day outcome. We have a rigorous approach in OBTT and the outcome code is not broken until all studies are completed. A full report on this agent will be provided in the next annual report.**



## Drugs# 6-10:

As indicated earlier in this report, we have just launched studies with treatment #6 which will be the



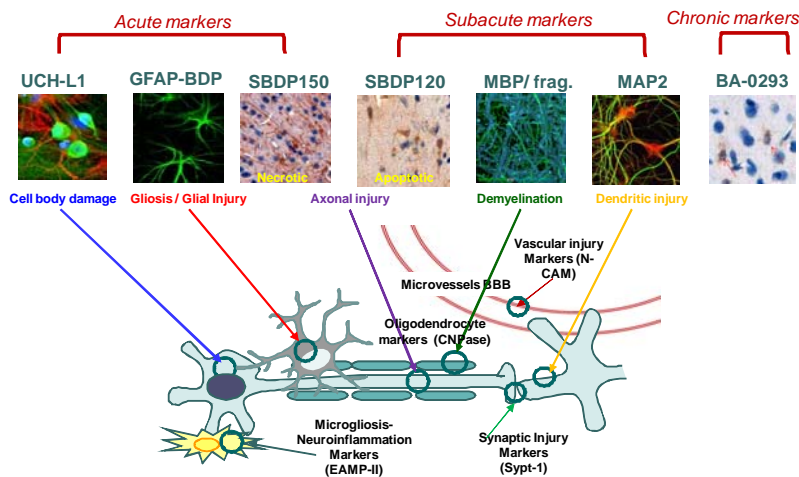
**Figure 13A-B.** Dose optimization tools for Minocycline which will be drug #7 in OBTT drug screening. Panel B shows the chromatographic assay to quantify Minocycline levels and its performance in the clinically relevant range of 50-500 ng/mL. Panel A shows the one-compartment predicted PK analysis for 30 mg/kg IV dosing in the rat. This approach could be extremely valuable to maximize therapeutic efficacy for some of the selected agents in OBTT Extended Studies.

sulfonylurea receptor-1 (SUR-1) antagonist glibenclamide (Glyburide). SUR-1 is implicated in the development of cerebral edema, and this antagonist has shown promise in several pre-clinical trials in stroke and TBI and is currently being tested in a small single center clinical trial that is funded by the Army (INTRuST Consortium). It is thus a very logical agent for testing in OBTT. Our first therapy in year 4 will be drug #7 which is Minocycline. We moved minocycline back from drug #6 to #7 behind glibenclamide because optimal dosing is still being worked out for minocycline whereas it has been clearly defined in preclinical studies for glibenclamide. Minocycline is a complex agent with a number of biological effects. Our pharmacology team has developed an assay for minocycline (**Figure 13A, B**), to assess serum and brain levels and we are currently measuring levels in **several dosing regiments**. **Our pharmacology team has been extremely helpful** with regard to the study design and drug preparation and administration for each therapy tested, and includes Drs. Samuel Poloyac, Philip Empey, and Travis Jackson at the University of Pittsburgh.

**Selection process for therapies proposed in 2014:** At the 2013 OBTT investigators' meeting at the National Neurotrauma Society Congress, based on literature review and recommendations of the investigators, oversight committee, and CCRP Programs, a total of 20 potential therapies were voted upon by our investigators. This included resveratrol, N-acetylcysteine, necrostatin, INO-1001, glibenclamide, AER 271, edaravone, melatonin, fluoxetine, amphetamine, DHA, head cooling, lithium, progesterone, intranasal NAD, MDL28170, VA-64, NIM-811, 8-OH-DPAT, and etanercept. A synopsis of the pre-clinical and clinical studies in TBI that have been performed with these therapies was provided by Dr. Kochanek and each investigator was also provided with key references on each therapy for review. A preliminary vote was taken prior to the meeting. At the meeting, after additional discussion, we selected the additional drugs for the upcoming year #4 for OBTT. Therapy #8 will be the CsA analog NIM 811—which blocks mitochondrial permeability transition pore formation, but unlike CsA is not immunosuppressive. Novartis has approved testing of this agent in OBTT. Drug #9 will be the brain penetrating antioxidant edaravone, which will also be evaluated in year 4. At the OBTT investigators' meeting, we selected two additional drugs for year 4 in anticipation of potential funding of our OBTT Extended Studies (OBTT-ES) application. We were asked by the CCCRP to submit that application for additional funding to test two more drugs per year taking further advantage of OBTT given our strong track record. As part of the process, we were asked to propose higher risk but possibly higher reward drugs in OBTT-ES. We chose the aquaporin-4 antagonist AER-271 targeting brain edema and the membrane resealing agent AQ-64 targeting cell death. More will follow on these therapies in next year's report. Dr. Kochanek has been in discussions with the investigators at Aeromics, the manufacturer of AER-271 and they have specifically indicated that they would be pleased and excited to supply the drug to OBTT for testing.

## Serum Biomarker Development and Application to the primary screening studies:

Banyan Biomarkers (Ronald Hayes, PhD) and the University of Florida (Kevin Wang, PhD)



**Figure 14.** Schematic of the Banyan biomarker portfolio. For OBTT in this report, the first two biomarkers UCHL-1 and GFAP are being used across the consortium.

For the biomarker studies, a rigorous sampling, shipping, and processing protocol has been followed since the inception of OBTT. Blood sampling was carried out at 4h, 24h and 21d, as described above. For the early time points, 0.7 mL was obtained. The final time point at sacrifice yielded 2-3 mL of blood obtained from the left cardiac ventricle via a 20-gauge needle. Blood was immediately placed in micro-centrifuge tubes and allowed to clot at room temperature for 60 min. Tubes were centrifuged at 5,000xg at room temperature for 5 min. Samples were collected, snap frozen on dry ice, and stored at -80°C until shipped. Each

sample was coded for rat number followed by a -4 h, -24 h, or final (-F) designation. Biomarker sampling that coincided with dosing was done before drug administration. Samples were shipped on dry ice and Banyan was notified. This approach produced high quality serum biomarker data across the OBTT consortium.

Our work has continued to focus on two biomarkers, a glial injury marker glial fibrillary acidic protein (GFAP) and a neuronal death marker UCHL1 (**Figure 14**). Based on prior experience, it was anticipated that the 4 h sample might represent largely the response to primary injury across models while the 24 h sample would reflect the evolution of secondary injury—influenced by both model and potentially by treatment. As shown in last year's report, we demonstrated extremely exciting results from the biomarker work in OBTT. In particular, GFAP appeared to perform well, with good correlations to hemispheric tissue loss—particularly in the setting of severe injury in CCI and PBBI. The results with GFAP also suggested some potential theragnostic value. Please see the detailed presentation of these findings in last year's OBTT annual report (namely Figures 25-34) which included 1) comparison of biomarker levels across models at 4 h after injury to compare the primary injury, 2) assessment of the effect of treatment on serum biomarker levels at 24 h after injury, and 3) assessment of the effect of treatment on differences between 4 h and 24 h (delta 24-4 h) levels. These comparisons are unique for the field of TBI. Please see last year's report for details.

A synopsis of the biomarker work in OBTT in this funding year is provided below and includes:

1. As detailed in the Table 6, Banyan has conducted a total of 982 biomarker assays from a total of 491 serum samples provided from three sites.

**Table 6: Summary of Biomarkers Tested**

KEY	W.O ID	Report Date	Total # of samples	Site	Biomarker tested		Total # of Assay
					UCHL-1	GFAP	
	R2912012	12/7/2012; 2/6/2013;	119	U. of Pitt	119	119	238
	R2922012	12/20/2012; 2/6/2013	121	U. of Miami	121	121	242
	R2932012	12/21/2012; 2/6/2013	251	WRAIR	251	251	502
	<b>Total</b>		<b>491</b>		<b>491</b>	<b>491</b>	<b>982</b>

2. Banyan conducted onsite training for sample collection at Virginia Commonwealth University (VCU) and shipment from VCU to the Banyan Service Laboratory. **This was done specifically to expand the biomarker work to include parallel biomarker assessments in the large animal micropig model.**

**This represents extremely exciting work. We will be able to directly compare the rat parasagittal FPI to the FPI injury in the micropig. In addition, we will now also be able to carry out a four model comparison of serum biomarkers. In addition, we will be able to carry out theragnostic studies in the micropig using serum biomarkers. All of these studies are unique to the field.** To insure quality control of shipment, a teleconference was held between the PI, Dr. John Povlishock and his post-doc, Audrey Lafrenaye, PhD, with Ron Hayes, Banyan OBTT PI, Dr. Stephen Larner and Banyan's Service Lab Director Jixiang Seaney. To date, 14 samples have been received by Banyan and are currently in storage awaiting the optimization and validation of Banyan's UCH-L1 and GFAP pig assays.

3. In support of the OBTT program, Banyan is also currently improving and optimizing UCH-L1 and GFAP assays.
  - a. Work is continuing to have the UCH-L1 and GFAP optimized for testing the porcine blood serum samples. The timetable is to have them available in the Service Laboratory by the end of November 2013. The samples will be analyzed shortly thereafter.
  - b. Banyan also continues to improve the UCH-L1 and GFAP rat assays both for LOD and LOQ. As part of this process Banyan is presently working to migrate all assays to a new platform that will allow Banyan to turnaround the sample assay testing more quickly and with improved accuracy. The goal is to eventually deliver improved results for biomarkers tested.
4. In consultation with OBTT PI's, additional assay improvements and optimization could be conducted on other biomarkers for the pig and/or the rat. SBDP150, MAP2 and S100 $\beta$  are three that are currently being examined.
  - a. SBDP150 is a byproduct of the cleavage of alpha II spectrin by calpain. This is a major response following TBI and any therapeutic treatment will need to take into consideration this action. Therapeutic treatments, especially those targeting calpain inhibitions, could benefit from analysis of this biomarker.
  - b. MAP2 is a more sub-acute biomarker that will allow for testing drug efficacy beyond 48 h. This will provide the consortium the opportunity to see if any of the drugs tested actually provide protection or just delays the normal negative response.
  - c. S100 $\beta$  is a biomarker that is considered one of the best biomarkers for TBI; unfortunately there is some concern that it may not be brain specific. However, that should not prevent its use at some point as it may become a biomarker that will be a good addition in a panel. It is our understanding the DoD has begun to show more interest in this biomarker based upon its potential.
5. The biomarkers listed in items 4a-c may also be added to the OBTT portfolio, particularly if the 1 h UCH-L1 time point data in our current studies do not prove to be fruitful. However, given that we have an established scoring matrix for all drug testing in OBTT, we will not alter the overall scoring so that we ultimately will be able to compare the scores for all drugs across all years in OBTT.

**It is noteworthy that the findings of cross model serum biomarker comparisons from OBTT have been selected for three oral presentations thus far at both the annual congress of the National Neurotrauma Society, and the MHSRS, including presentations made at the most recent 2013 meetings of both of these organizations. Note that at these meetings, oral abstract selection is highly competitive. Our biomarker findings are thus being viewed as important by the field. We believe that they have relevance to both pre-clinical drug screening and also for a better understanding of serum biomarkers in clinical use—since clinical TBI is often highly complex and heterogeneous.**

In contrast to the strong performance of GFAP, UCH-L1 as assessed at 4 h did not show major injury effects across models, possibly because of its short half-life. Based on this finding, we added a 1 h sampling time point (and thus now sample at 1 h, 4 h, 24 h and 21 d for all biomarker assessments specifically to theoretically improve the utility of UCH-L1. That time point for sampling was used beginning with the studies of Levetiracetam which is nearly completed and the results are ongoing. In the interim, we are also using this more comprehensive sampling approach (including a 1 h time point) in the next study with glibenclamide (Glyburide). As discussed above, we are also beginning to explore the potential use of additional serum biomarkers across the studies in the OBTT consortium.

#### **OBTT Studies in a large animal model of TBI: FPI in micropigs:**

*John Povlishock, PhD, Site PI, Virginia Commonwealth University*

During the past funding period (i.e., in year 3), 14 micropigs were subjected to mild/moderate TBI, with some of the animals equipped with cranial windows to assess vascular function. All animals were processed for immunocytochemistry to assess the burden of axonal damage. To improve the fidelity and rigor of axonal counting, the employed APP antibodies were tagged with fluorophores and the images digitally acquired and converted to a grey scale to allow for computer-assisted quantitative analysis. In this fashion, analyses of the corpus callosum, thalamus, and superior colliculi, all zones involved in human TBI, showed a striking yield of large numbers of damaged axons per unit area, with counts ranging as high as 85 damaged axons per 5 mm<sup>2</sup>. In concert with assessments of microvessel reactivity and axonal quantification, 7 of these same animals were evaluated for biomarker screening, with blood samples consistently harvested pre-injury, post-TBI surgery/pre-injury, immediately post-TBI, and at 30 min, 1 h, 3 h, and 6 h post-TBI time points. The samples were processed consistent with a protocol established by Banyan Biomarkers and all were shipped to Banyan for analysis (as described previously in this report), moving on the premise that these axotomy-laden animals would constitute an excellent source for detailed biomarker analysis targeting diffuse axonal injury (DAI).

Lastly, to further strengthen the biomarker and axonal count data, parallel immunocytochemical screening was performed on the same tissue samples used to evaluate the burden of axonal injury to assess UCH-L1 and Iba-1 immunocytochemistry. These samples were performed to confirm the upregulation of UCH-L1 in the sites of axonal injury while also providing another marker of the burden of axonal damage via the dramatic activation of Iba-1<sup>+</sup> cell types. While these immunocytochemical studies require further quantitative analyses, it is of note that those nuclear regions with axonal damage reveal a dramatic upregulation of UCH-L1 within the neurons found in the same field, together with the presence of numerous Iba-1 reactive microglia, which in our estimation confirm the involvement/upregulation of both markers following diffuse injury.

All of these studies were carried out to set the stage for definitive drug testing when the best agent is identified in the rat studies to be moved up to phylogenetic scale to the micropig model. **Also, as discussed previously the biomarker work is allowing unique cross model comparisons not only for FPI, but across all of the four models being used in OBTT, both for direct model comparisons across the various pathologies and for theragnostic purposes.**

#### **Other accomplishments by the OBTT consortium in year 3:**

1. The OBTT consortium investigators are working on 6 manuscripts that will comprise a special issue of the *Journal of Neurotrauma* reporting the results of the first 3 therapies and the biomarker work thus far in OBTT.
2. Dr. Kochanek represented OBTT at the recent US Army Neurotrauma Pharmacology Workshop, which generated a comprehensive document for the Army on TBI pharmacology that was just published in the *Journal of Neurotrauma*.

**KEY RESEARCH ACCOMPLISHMENTS Since THE INCEPTION OF OBTT—Accomplishments for this funding year are bolded for convenience of the reviewer.**

1. **IACUC and ACURO Approval at all sites along with necessary updates**
2. **Creation and continual updating of an Operations Manual for the OBTT consortium by Dr. Kochanek**
3. **Monthly consortium investigator conference calls**
4. **TBI drug therapy literature review, investigators survey, and selection of the first two therapies to be evaluated by the OBTT consortium**
5. **Comprehensive review of the TBI literature for the first nine drugs, nicotinamide, EPO, CsA, Simvastatin, Levetiracetam, Glibenclamide, Minocycline, NIM-811, and Edaravone by Dr. Kochanek, with updating of the manual through the most current agent (IACUC and ACUROs either submitted or approved at all sites).**
6. Publication of a manuscript on the OBTT concept in the *Journal of Trauma* (1)
7. Presentation of five abstracts on the individual components of OBTT to the 2011 ATACCC meeting. Those abstracts served as the basis of a symposium at the conference.
8. Report sent by Dr. Kochanek on the launching of OBTT to the Therapy and Oversight Committee and Consultants

9. Completion of all experiments for drugs #1 (nicotinamide), #2(EPO), and #3 (CsA)—in primary screening across three rodent models. And complete analysis of all data on drugs #1 and #2, with #3 in process.
10. Investigators meeting held on at the 2011 and 2012 National Neurotrauma Society Meeting
11. Presentation of an afternoon symposium on OBTT by the PI and site PIs at the 2011 ATACCC conference, and a plenary lecture on OBTT by the PI at the 2012 MHSRS conference.
12. Presentation by the PI of a plenary lecture on OBTT at the 2012 annual meeting of National Neurotrauma Society.
13. Presentation of two abstracts by site PIs at the 2012 meeting of the National Neurotrauma Society.
14. Re-establishment and continued refinement of the large animal micropig model of FPI TBI at Virginia Commonwealth University
- 15. Dr. Kochanek also represented OBTT at the US Army Neurotrauma, Pharmacology Work Group. He was the second author of the comprehensive document generated by that group and recently published in the *Journal of Neurotrauma*.**
- 16. Presentation of 7 abstracts at the 2013 meeting of the National Neurotrauma society. Six of these were posters and one was an oral presentation that was related to the biomarker work.**
- 17. Presentation of 2 posters at the 2013 MHSRS conference.**
- 18. An oral presentation on the results of the biomarker work in OBTT at the 2013 MHSRS.**
- 19. Preparation of a full grant application titled Operation Brain Trauma Therapy-Extended Studies requested by CCCR. Dr. Kochanek prepared the application.**
- 20. Ongoing preparation of six manuscripts by the OBTT investigators for invited submission as a special issue of the *Journal of Neurotrauma* devoted to OBTT.**

**REPORTABLE OUTCOMES (All reportable outcomes since project inception are shown, those from 2013 are shown in bold font)**

1. Kochanek PM, Bramlett H, Dietrich WD, Dixon CE, Hayes R, Povlishock J, Tortella F, Wang K: A novel multi-center pre-clinical drug screening and biomarker consortium for experimental traumatic brain injury: Operation Brain Trauma Therapy. *J Trauma* 71(1 Suppl):S15-24, 2011.
2. Kochanek PM, Dixon CE: Operation Brain Trauma Therapy (OBTT) Consortium: Program Overview/University of Pittsburgh Program. Presented at the ATACCC Meeting, Ft. Lauderdale, Florida, August 15-19, 2011.
3. Bramlett HM, Dietrich WD. Operation Brain Trauma Therapy (OBTT) Consortium: University of Miami Miller School of Medicine Program. Presented at the ATACCC Meeting, Ft. Lauderdale, Florida, August 15-19, 2011.
4. Shear DA, Schmid KE and Tortella FC. Operation Brain Trauma Therapy (OBTT) Consortium: The WRAIR Program (Penetrating Ballistic-Like Brain Injury). Presented at the Advanced Technology Applications to Combat Casualty Care (ATACCC) Conference in Fort Lauderdale, FL, 2011.
5. Povlishock, JT. Operation Brain Trauma Therapy: The Virginia Commonwealth University Program. Presented at the Advanced Technology Applications to Combat Casualty Care (ATACCC) Conference in Fort Lauderdale, FL, 2011.
6. Kevin K.W. Wang, Ronald L. Hayes Operation Brain Trauma Therapy (OBTT) Consortium: Banyan Biomarkers Core. Presented at the Advanced Technology Applications to Combat Casualty Care (ATACCC) Conference in Fort Lauderdale, FL, 2011.
7. Kochanek PM: Operation Brain Trauma Therapy (OBTT). Oral plenary presentation, 2012 Congress of the National Neurotrauma Society, Phoenix, AZ, July, 2012.
8. Kochanek, Patrick M.; Bramlett, Helen; Dixon, C. Edward; et al. Cross model comparison of behavior, neuropathology, and serum biomarkers after controlled cortical impact, parasagittal fluid percussion, and penetrating ballistic-like brain injury: results from Operation Brain Trauma Therapy. *J Neurotrauma* 29:10, A23-A23, 2012.

9. Mondello, Stefania; Bramlett, Helen M.; Dixon, C. Edward; et al. Differential effect of nicotinamide on serum damage marker profiles following controlled cortical impact, parasagittal fluid percussion, and penetrating ballistic-like brain injury: results from Operation Brain Trauma Therapy. *J Neurotrauma* 29:10, A48-A48, 2012.
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14. Mondello, Stefania; Bramlett, Helen M.; Dixon, C. Edward; et al. Differential effect of nicotinamide on serum damage marker profiles following controlled cortical impact, parasagittal fluid percussion, and penetrating ballistic-like brain injury: results from Operation Brain Trauma Therapy. *Proceedings of the MHSRS, Ft. Lauderdale, FLA, August, 2012.*
15. Kochanek PM: Operation Brain Trauma Therapy (OBTB). Oral plenary presentation, *Proceedings of the MHSRS, Ft. Lauderdale, FL, August, 2012.*
16. **Shear DA, Deng-Bryant Y, Pedersen R, Sun J, Mondello S, Wang KKW, Hayes RL, Schmid KE, Tortella FC. Operation Brain Trauma Therapy Consortium: Dose-Response Evaluation of Cyclosporine A in the WRAIR Penetrating Ballistic-Like Brain Injury (PBBi) Model. *J Neurotrauma* 30: A170-A171, 2013.**
17. **Shear DA, Pedersen R, Sun J, Mondello S, Wang KKW, Hayes RL, Schmid KE, Tortella FC. Operation Brain Trauma Therapy Consortium: Dose-Response Evaluation of Erythropoietin in the WRAIR Penetrating Ballistic-Like Brain Injury (PBBi) Model. *J Neurotrauma* 30: A171, 2013.**
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## CONCLUSION

The unique multicenter pre-clinical drug screening consortium OBTT continues to be highly productive and has just begun screening its 6<sup>th</sup> therapy and carrying out studies to optimize therapy #7 for the consortium. In addition, exciting biomarker applications have also been successfully launched and those data have generated valuable findings. Work has also now included biomarker studies in the large animal micropig model. An outstanding collaboration between civilian and US Army investigators has been successfully developed. The consortium data have generated some of the first cross-model comparisons in the field of experimental TBI. Surprisingly, the findings of OBTT suggest that the literature is somewhat inflated with regard to the efficacy of various therapies in pre-clinical models of TBI. Of note, that consideration has not previously been listed as one of the reasons for the failure of clinical trials to date. Thus, if a therapy is shown by OBTT to have robust and reproducible effects across the consortium, we believe that it would represent a potential breakthrough agent, and would be very deserving of fast tracking to clinical trials. The work has been presented at major national meetings in the field and for the DOD and the consortium's findings have been well received. Overall, no significant problems have been encountered and our team is highly collaborative and productive.

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